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MINIREVIEW

Origin and Production of Acetoin during Wine Yeast Fermentation

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INTRODUCTION

The flavor of alcoholic beverages is produced by a very large number of compounds (50). Among these, acetoin is important because of its involvement in the bouquet of wine, and it is the key compound in the biosynthesis of 2,3-butanediol and diacetyl. These two compounds are closely related to acetoin, representing three levels of oxidation in one four-carbon skeleton (8). Their contribution to the aroma and flavor of wine is not easily assessed. Neither 2,3-butanediol nor acetoin is strongly odorous; in fact, their threshold values in wine are very high, both being about 150 mg/liter (19, 72); however, diacetyl, which creates an off flavor in alcoholic beverages, has a characteristic odor and is detectable in wine at very low levels (threshold value, 8 mg/liter) (19). In this picture, the flavor significance of acetoin is more likely to be attributable to its potential aroma than to the odor itself.

Acetoin is formed during fermentation by the microbial activity of lactic acid bacteria and yeasts. Therefore, the study of acetoin in wine is an integral part of investigation of the influence of microorganisms on the composition and quality of wine.

ACETOIN PRODUCTION IN WINE YEASTS

There are few exhaustive studies reporting the amounts of acetoin produced by individual species during wine fermentation. Wine yeasts were subdivided by Antoniani (2) into actively fermenting yeasts, producing only 2,3-butanediol, moderately fermenting yeasts, producing both 2,3-butanediol and acetoin, and weakly fermenting yeasts, producing only acetoin.

In general, it is assumed that the yeasts of the genus *Saccharomyces* do not produce significant amounts of acetoin by the end of fermentation (31). Haukeli and Lie (28), studying 13 *Saccharomyces* strains, found an acetoin content ranging from 2.4 mg/liter for a strain of *Saccharomyces sake* to 40 mg/liter for a strain of *S. carlsbergensis*. Acetoin is produced by *S. cerevisiae* in the early phase of fermentation, reaching its maximum of 25 to 100 mg/liter at about the midway point, and then its content declines rapidly in the final stage of the process, presumably as a result of reduction to 2,3-butanediol (26, 31). Consequently, normal dry wines fermented by *S. cerevisiae* generally contain acetoin but at low levels. A biometric study of 100 strains of *S. cerevisiae* (61) showed that most strains exhibit uniform behavior, producing acetoin from nondetectable amounts to 12 mg/liter, indicating that low acetoin production is the dominant pattern in this species (Fig. 1). Four strains were found to produce abnormally large amounts of acetoin, up to about 200 mg/liter in grape must. Therefore, high levels of acetoin, sometimes determined in natural wine fermentation and attributed to bacterial action (26), might be caused by these yeasts.

After the first studies by Antoniani and Gugnoni (3), reporting that two apiculate yeast strains produced, respectively, 100 and 200 mg of acetoin per liter of wine, it has generally been assumed that acetoin is normally produced in large amounts by apiculate yeasts. Romano et al. (63), studying 96 strains of *Kloeckera apiculata* and *Hanseniaspora guilliermondii* for their ability to produce acetoin in synthetic medium and in must, confirmed that a high level of acetoin is a common characteristic of both species (Fig. 1). A large interstrain variability was found, indicating that formation of this compound is also a strain characteristic in apiculate yeasts. Acetoin produced by apiculate yeasts, the dominant yeasts of the early stage of must fermentation (for a review, see reference 20), is utilized by *S. cerevisiae*, the yeast completing the fermentation process. *S. cerevisiae* seems to be able to utilize the acetoin produced by apiculate yeasts to form 2,3-butanediol (3) or to increase ethanol production or other secondary products (32, 86).

In a study of acetoin production in 70 strains of the genus *Zygosaccharomyces* isolated from grape must, Romano and Suzzi (62) showed that the two species studied, *Zygosaccharomyces bailii* and *Z. fermentati*, behave rather differently from each other (Fig. 1). Most of the *Z. bailii* strains produce more than 25 mg of acetoin per liter, whereas a few strains of *Z. fermentati* form detectable amounts of the compound (2 mg/liter).

Table 1 reports acetoin production by the above-mentioned yeasts and further species that can be present in must fermentation, such as *Candida stellata*, *Torulaspora delbrueckii*, and *Saccharomycodes ludwigii*. In particular, Fig. 1 also shows histograms of acetoin production in 30 strains of *Saccharomycodes ludwigii* (unpublished data). This species is a high acetoin producer, with the majority of the strains forming 100 to 200 mg/liter.

ACETOIN CONCENTRATIONS IN WINE

Acetoin is a normal product of alcoholic fermentation (56), and its content in wine can originate from different sources: yeasts during alcoholic fermentation, spoilage yeasts, bacteria during malolactic fermentation, and spoilage bacteria. The amounts of acetoin can vary in wine, generally up to 80 mg/liter. Ribéreau-Gayon and Peynaud (58) reported ranges of acetoin of 4 to 25 mg/liter. Peynaud and Lafon (56) found a content of 2 to 20 mg/liter in table wines, and Postel and

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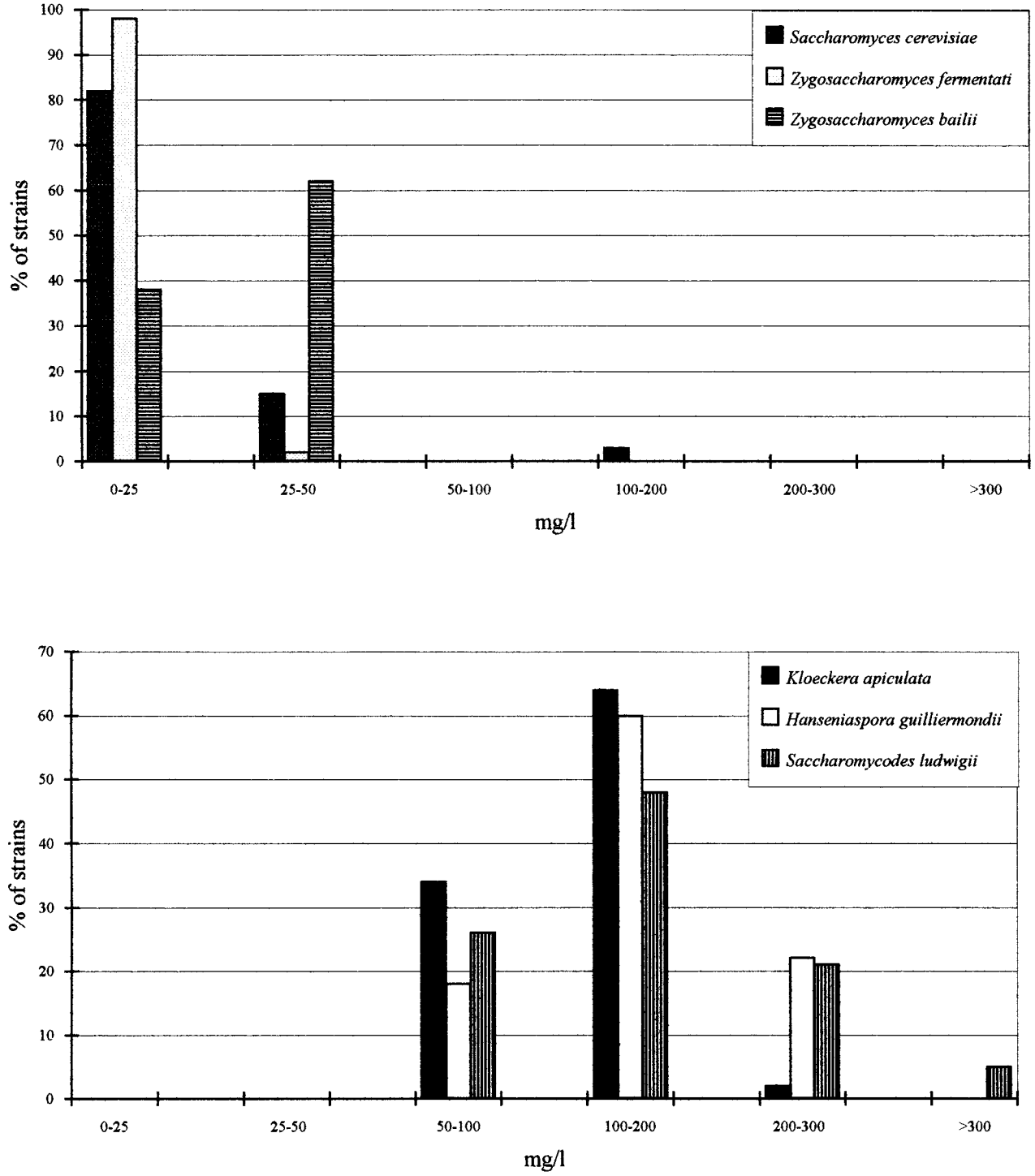

FIG. 1. Acetoin production in grape must by different natural wine yeasts.

Guvene (57) found a content of 2 to 32 mg/liter. In general, the red wines (46 mg/liter) show considerably higher contents of acetoin than the white wines (12 mg/liter). The general levels of acetoin found in wines from different countries are listed in Table 2.

The acetoin content of sweet (dessert) wines produced by the addition of wine spirits to half fermentation was much higher (50 to 200 mg/liter) than that of the corresponding dry wines produced by complete fermentation (about 5 to 20 mg/liter) (26). Significantly higher levels of acetoin (up to 450 mg/liter) were found in sherry samples (10).

In wines which have not undergone malolactic fermentation, the acetoin content is generally low. Fornachon and Lloyd (21)

TABLE 1. Acetoin produced in wine by different species of wine yeasts

Species	No. of strains	Acetoin production (mg/liter)		Reference
		Range	Mean	
Saccharomyces cerevisiae	1	0–0		3
	10	26.3–76.2	56.4	67
	100	0.1–194.6	8.5	61
Torulaspora delbrueckii	10	0.0–21.9	5.5	67
Kloeckera apiculata	2	90–220		3
	10	68.6–225.0	138.1	67
	48	55.8–187.4	290.6	65
Hanseniaspora guilliermondii	48	50.3–258.1	168.3	65
Hanseniaspora valbyensis	10	22.9–408.7	144.9	67
Candida stellata	10	34.9–254.1	94.9	67
Zygosaccharomyces bailii	35	17.1–34.4	24.8	62
Zygosaccharomyces fermentati	35	0.0–0.0	0.0	62
Saccharomycodes ludwigii	10	211.7–478.3	310.2	67
	20	55.5–362.7	154	Fig. 1

found values of 0.7 to 0.9 mg/liter, and Dittrich and Kerner (18) found 1.5 to 5.9 mg/liter.

ACETOIN BIOSYNTHESIS IN YEASTS

The presence of carbonyl compounds, such as diacetyl and acetoin, in fermented products was once thought to be due entirely to bacterial action. However, in the 1950s and 1960s, it became clear that yeasts also produce acetoin and diacetyl (15, 36, 37, 44, 52). Nevertheless, the mechanism of production remained controversial for a long time until the studies of Collins (8) and Wainwright (83) revealed the biosynthesis of acetoin in yeasts. Figure 2 gives a summary of the reactions that are actually known.

In wine yeasts, acetoin is a significant by-product of carbohydrate metabolism, which occurs only in the presence of fermentable carbohydrate or of pyruvic acid, not otherwise needed in the metabolism of the microorganisms (27, 49), particularly in the synthesis of cell materials. Yeast forms pyruvate from glucose by glycolysis, and a key reaction in the utilization of pyruvate is its decarboxylation to hydroxyethylthiamine PP_i, called the acetaldehyde-TPP complex (active acetaldehyde), by means of thiamine PP_i (TPP). Depending on the substrate with which active acetaldehyde reacts, the following sequences can be derived:

Active acetaldehyde + pyruvate → acetolactate → acetoin Pathway A
Active acetaldehyde + acetyl coenzyme A → diacetyl → acetoin Pathway B
Active acetaldehyde + acetaldehyde → acetoin Pathway C

Pathway A

The synthesis begins with pyruvate, which is derived from carbohydrate degradation. Active acetaldehyde and pyruvate are then transformed into α-acetolactate by means of acetohydroxy acid synthetase. Yeasts produce some α-acetolactate (8), and the formation is certainly enzymatic, but α-acetolactate is also formed in nonenzymatic model systems from pyruvate and thiamine.

Yeasts belonging to the genus *Saccharomyces* produce α-acetolactate in considerable amounts during fermentation, and this highly labile compound may easily be converted to diacetyl or to acetoin, particularly in the presence of oxygen (28). An oxygen-poor medium (0.2 mg of O_2 per liter) leads to the most rapid α-acetolactate formation, and maximum levels are obtained in an overaerated medium (17.4 mg of O_2 per liter). Normal aeration leads to lower levels. The oxygen-dependent formation of α-acetolactate is closely related to the utilization of carbohydrates, the amino acids threonine and lysine, and the potassium and iron cations present in the medium (for a review, see reference 24).

Collins (8) reports that yeasts produce α-acetolactate, but they do not have α-acetolactate decarboxylase and cannot form acetoin from the α-acetolactate they produce (7, 36). According to Chuang, Collins, and Juni, nature apparently has made sure that yeasts will use the α-acetolactate they produce exclusively to make products that are important in their metabolism, such as valine and pantothenic acid. The failure of valine to prevent the formation of acetoin by a strain of *S. cerevisiae* is consistent with results showing that α-acetolactate does not serve as an intermediate in the synthesis of acetoin in this organism (7). However, by heat treatment under strictly anaerobic conditions, commercial α-acetolactic acid can be directly converted to acetoin nonenzymatically at a rate of about 0.05% h^{-1}, yielding primarily acetoin with some diacetyl (13, 34, 77). A maximum of 80% acetoin conversion was obtained from the fermenting medium by 30 min of anaerobic (dissolved oxygen concentration, <0.1 mg/liter) heat treatment at 70°C (35). However, microbial α-acetolactic acid is decarboxylated to acetoin only in the presence of the enzyme α-acetolactic acid decarboxylase. An explanation of this observation is that the α-acetolactic acid produced by microorganisms is not free but is completely bound to enzymes, so that it cannot be lost by spontaneous decomposition.

TABLE 2. Acetoin concentrations in wine from different countries

Country	Wine type	Acetoin concn (mg/liter)		Reference
		Range	Mean	
Italy	Red table	3–28	6.6	25
	White table	0.1–19.9		53
	Red table	0.1–24.0	19.2	53
France	Miscellaneous	2–84	10	59
	White table	4.5–12	7	55
		16–129		4
	Red table	6–18	11	55
		55–261		4
Germany	Miscellaneous	0–29	7.8	1
	White table	1.9–31.7	5.9	57
	Red table	5.9–38.2	15	57
Russia	Table	8–36.2	15.1	38
Japan	Table	6–140	16–53	72
Australia	White table	0.7–4.3	1.8	21
	Red table	1.5–44	10.6	21
United States	White table	17.4–20.4	18.3	26
	Red table	18.8–20.3	19.6	26

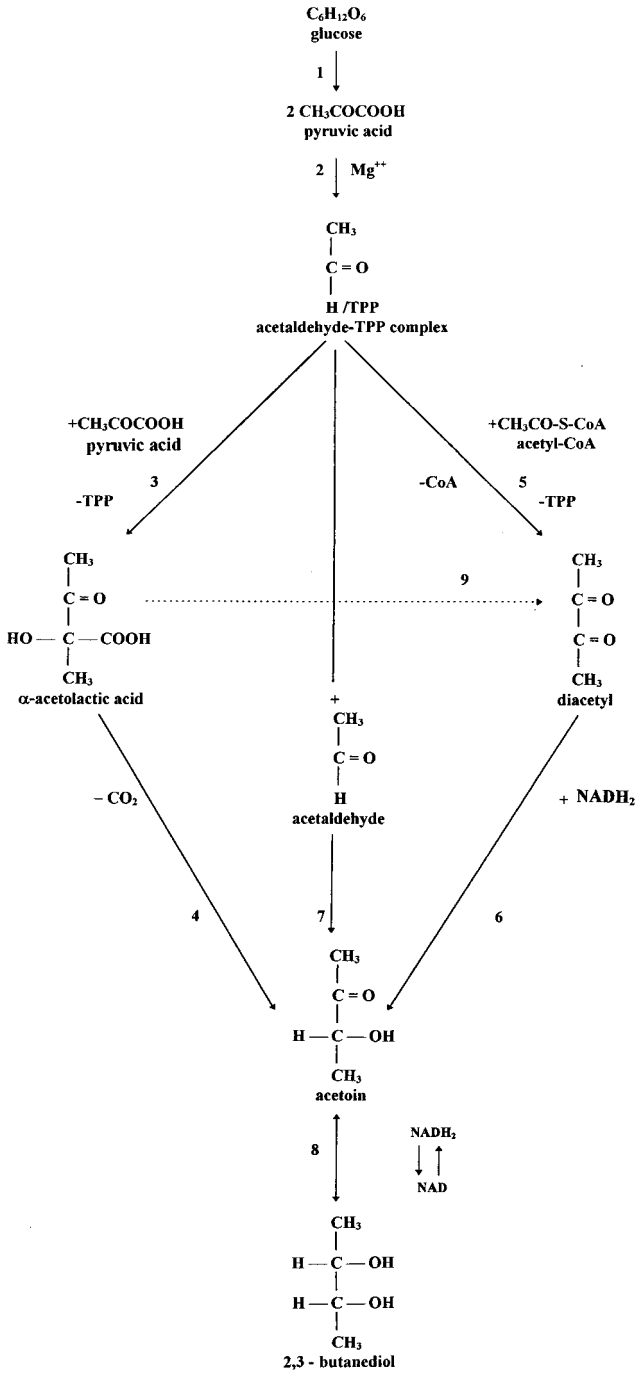

FIG. 2. Scheme of acetoin biosynthesis in yeasts. 1, Glycolysis; 2, pyruvate decarboxylation; 3, acetohydroxy acid synthetase; 4, α-acetolactate decarboxylase; 5, diacetyl synthetase; 6, diacetyl reductase; 7, acetaldehyde condensation; 8, acetoin reductase; 9, oxidative decarboxylation. CoA, coenzyme A.

Contrary to what has hitherto been supposed, it has been demonstrated that yeasts can also decarboxylate α-acetolactate directly to acetoin (28) by the mechanism normally used in bacteria. In radioactivity trials, Deiana et al. (12) found that some strains of *Debaryomyces hansenii* produced acetoin from glucose and lactic acid through the decarboxylation of α-acetolactate. The acetoin produced was affected by the strain, substrate, and time of incubation.

α-Acetolactate can be degraded nonenzymatically into diacetyl by means of oxidative decarboxylation, and its decomposition depends on the physical and chemical properties of the alcoholic beverages. The conversion of α-acetolactic acid to diacetyl is quantitative if warm solutions of α-acetolactate are shaken with air (84). The overwhelming evidence is that diacetyl is formed from α-acetolactic acid as a by-product in the biosynthesis of α-oxo-isovaleric acid, an intermediate in the biosynthesis of valine, leucine, and pantothenic acid (76, 83).

Finally, the conversion of α-acetolactic acid to diacetyl generally takes place in a nonenzymatic manner outside the yeast cell, and further data are needed to prove that yeast enzymes or intact cells catalyze this change.

Pathway B

Another route for acetoin biosynthesis in yeasts is the condensation of active acetaldehyde with acetyl coenzyme A to form diacetyl (7), which is successively reduced to acetoin. Although they produce a large amount of acetoin (7), some strains of *S. cerevisiae* are unable to produce diacetyl because of the inefficiency of diacetyl synthetase, the enzyme necessary for the reaction of acetyl coenzyme A with active acetaldehyde. The amount of diacetyl synthetase in the cells of *S. cerevisiae* is influenced by the age of the culture and by the composition of the medium, specifically, the glucose content (8). Aeration of cultures increases the production of diacetyl.

Once yeasts have formed diacetyl by this mechanism, they can, by means of diacetyl reductase, reduce diacetyl to acetoin (71, 75), and many of them can reduce the acetoin to 2,3-butanediol (31, 36). Diacetyl reductase catalyzes the reduction of diacetyl to acetoin with NADH as an electron donor but fails to catalyze the reverse reaction (70), demonstrating that reduction of diacetyl by the enzyme is irreversible, whereas 2,3-butanediol is oxidized to acetoin with NAD^+, demonstrating that the reduction of acetoin is a reversible reaction.

Diacetyl reductase is most active on diacetyl at pH 7.0, whereas it is not very active at low pH values (69); consequently, there is less conversion of diacetyl to odorless compounds when the pH of a culture is low. The enzyme is most active at 40°C in *Kluyveromyces marxianus* (70) and in *S. carlsbergensis* (46). Diacetyl reductase activity has been detected in *S. cerevisiae* (30), *S. carlsbergensis* (43, 46), and *Candida utilis* (80).

Actively metabolizing yeasts rapidly reduce diacetyl to acetoin and then to 2,3-butanediol, and this rapid metabolism explains the absence of appreciable amounts of diacetyl during the active phase of fermentation. The more yeast that is present, the more readily diacetyl is reduced, so that high inoculum rates favor diacetyl reduction. The reduction is faster at higher temperatures and if the yeast is uniformly suspended in the medium (5, 24).

Pathway C

Yeasts also form acetoin by a mechanism different from that observed in bacteria: by condensation of the active acetaldehyde (acetaldehyde-TPP complex) with free acetaldehyde formed from pyruvate (36), without the intermediate formation of α-acetolactate. The production of acetoin by cell extracts of the yeast is stimulated by the addition of acetaldehyde, which indicates that yeast forms acetoin by the condensation of free acetaldehyde with the acetaldehyde-TPP complex. This mechanism, described by Chuang and Collins (7), confirms that acetoin cannot be oxidated to diacetyl, contrary to previous reports (42, 68, 78).

The extent of acetoin formation depends on the $NAD^+/(NADH + H^+)$ ratio and the intracellular concentration of pyruvic acid (16, 17). Apparently, the reaction steps leading from pyruvic acid to acetoin and diacetyl are inhibited by copper (39), most likely by preventing the formation of the common intermediate active acetaldehyde (39). From active acetaldehyde, *S. cerevisiae* and *Schizosaccharomyces pombe* form both acetoin and free acetaldehyde (16).

FACTORS AFFECTING ACETOIN PRODUCTION

Many winemaking variables affect acetoin production by yeasts. The production of acetoin is dependent on temperature, increasing greatly as the fermentation temperature rises (51). When the temperature increases, the rate of α-acetolactate decomposition in the fermentation medium also increases, and more diacetyl and then acetoin are produced (23, 66). A fermentation temperature of 30°C, in comparison with 12, 18, and 24°C, greatly increased the acetoin produced in wine by *S. cerevisiae* (60). Inoue (33) found that more acetohydroxy acids were formed when the fermentation temperature was elevated. This case would suggest a preferential route of acetoin production from acetolactate.

Acetoin is found in unusually large amounts in certain strongly aerated yeast fermentations and wines (10). It is formed in the range of 6 to 8 mg/liter under anaerobic conditions and 220 to 370 mg/liter under aerobic conditions. The redox potential of the fermenting medium seems to be an important factor in controlling the decomposition of the acetohydroxy acids (34, 85). At higher levels of aeration, large quantities of acetoin are produced (9). Aeration conditions which favor the accumulation of acetoin also increase the formation of higher alcohols, especially isobutyl alcohol and, to a lesser extent, isoamyl alcohol. Peynaud (54) pointed out that prolonged aeration of wine does not increase the level of acetoin in the absence of microbial activity. Significant amounts of acetoin are found in some cultures of "flor" sherries (10).

Acetoin production is affected by substrate. Deiana et al. (12) reported that diacetyl and acetoin production was significantly influenced by the substrate, with larger amounts forming when the substrate was glucose. While confirming the influence of medium composition on the quantity of acetoin produced by yeasts, Romano and Suzzi (60) recorded a smaller quantity of acetoin in grape juice and wort compared with that in the synthetic medium. The effect of medium composition, including the valine concentration, is usually indirect and is related to the rate and extent of yeast growth (84).

The production of acetoin depends on the yeast strain. Romano and Suzzi (61) found that the different strains of *S. cerevisiae* significantly influence the formation of acetoin in the various wines. The existence of well-defined behavior within each production class (low, medium, and high acetoin producers) allowed the designation of certain strains as consistently high or low producers of acetoin.

The influence of the yeast strain is dependent on the inoculum rate used, which can vary between normal (5 g of wet yeast per liter) to twice normal or more. The effect of inoculum rate on diacetyl and consequently on acetoin formation has been widely investigated in brewery fermentation, with somewhat contradictory results. However, the predominant opinion tends to support the view that increased inoculum rates yield increased diacetyl and acetoin formation (24).

The conversion of α-acetolactate to diacetyl occurs nonenzymatically outside the yeast cell and is dependent on pH. The reaction has an optimum at the pH of beer (about 5) (29), and low pH results in faster degradation of α-acetolactate to diacetyl (6). In contrast, diacetyl reductase, the enzyme converting diacetyl to acetoin, is not very active at low pH (69); consequently, there would be less conversion of diacetyl to odorless compounds when the pH of a culture is low.

TABLE 3. Segregation of the character "high acetoin production" in *S. cerevisiae*

Parental strain	Gene	No. of tetrads with *ACR*/*acr* ratio of:				
		4:0	3:1	2:2	1:3	0:4
Low producer	*ACR*	8	0	0	0	0
High producer	*acr*	0	0	0	0	10
Hybrids						
XPG5(12)	*ACR*	0	1	8	0	0
XPG5(23)	*ACR*	0	0	8	0	0
XPG5(29)	*ACR*	0	0	8	1	0

The addition of potassium sulfite or metabisulfite to alcoholic beverages can remove the odor and flavor due to diacetyl by forming a complex with it, and there have been suggestions that the addition of these reducing compounds removes diacetyl by converting it to acetoin and 2,3-butanediol (83).

A direct relationship between the acetoin content and the age of wine does not always seem to exist (25), despite the higher oxidation-reduction potential of older wines. Differences in the amount of oxygen reaching the wines during aging might account for the lack of correlation.

Finally, when the individual technological fermentation conditions are compared, the oxygen content and the inoculum rate exert the greatest influence, after the yeast strains, on the formation of acetoin and related compounds. The fermentation temperature plays a less significant role.

GENETICS OF ACETOIN PRODUCTION

Taking into account the fact that acetoin has a potential influence on the aroma of alcoholic beverages, an understanding of the genetic basis of its production could be useful for the modulation of the flavor of the final product.

Little information is available on the genetics of acetoin production in wine yeasts, whereas several reports deal with brewing yeasts. Genetic studies on the production of carbonyl compounds have focused particularly on the selection or construction of strains with a reduced or mutationally impaired catalytic function of α-acetohydroxy acid synthase (22, 82) to achieve subthreshold levels of vicinal diketones (diacetyl and 2,3-pentanedione), which are considered to be very unpleasant in finished beer. To overcome the diacetyl problem, studies on *ILV* (isoleucine/valine/leucine) genes were performed (11). The isomeroreductase (ILV5) activity causes the accumulation of the acetohydroxy acids, direct precursors of the vicinal diketones. In particular, an amplification of *ILV5* resulted in a 70 to 80% decrease in total vicinal diketone levels without any further change in the organoleptic profile (14, 81). The cloning of α-acetolactate decarboxylase from *Klebsiella terrigenis* and its successful expression in brewer's yeast were reported by Simon et al. (73, 74). Recombinant brewer's yeasts with α-acetolactate decarboxylase enzyme activity were constructed (45, 76), and the α-acetolactate decarboxylase enzyme produced by the transformant, catalyzing the direct formation of α-acetolactate to acetoin (40), decreased the total diacetyl content.

The genetic basis of acetoin production in *S. cerevisiae* wine yeasts was determined by crossing strains forming different amounts of this compound (64). Table 3 reports the segrega-

tion of the characteristic "high acetoin production" in tetrads of two parental strains, whose spores all had high levels (average, 295.4 mg/liter) or low levels (average, 10.7 mg/liter) of acetoin. Crosses between strains that differ in acetoin production always exhibited a low level of acetoin, and the tetrad analysis of the hybrids showed that high versus low acetoin production is segregated as a single gene. This suggests that high levels of acetoin (recessive character) are due to mutation in a gene coding for a presumptive repressor that normally keeps acetoin at low levels. The production of 2,3-butanediol in must by high- and low-acetoin-producing strains differed significantly, resulting in an inverse correlation between the acetoin and 2,3-butanediol levels. In each tetrad, the two low acetoin producers yielded the highest levels of 2,3-butanediol whereas the two high producers yielded the lowest levels of 2,3-butanediol, suggesting a leaky mutation in acetoin reductase of the low 2,3-butanediol-producing strains (63). The occurrence in nature of high-acetoin-producing strains of *S. cerevisiae* could be described as a possible succession of events leading to the replacement of one population by another through a process called genome renewal (48). Most natural wine yeast strains are diploid and are homozygous for the homothallism gene *HO*. They can accumulate heterozygous mutations (*ACR*/*acr*). Sporulation of such diploids yields spores that become homozygous diploids (*ACR*/*ACR*, *acr*/*acr*), some of which may replace the original diploid. This process explains the appearance of natural wine yeasts possessing the character "high acetoin production" (*acr*/*acr*).

CONCLUSIONS

This account has outlined acetoin formation in wine yeasts and the factors (strain, genetics, biochemical factors, and cultivation factors) that influence its concentrations in wine. The role of acetoin in wine is significant both as a precursor of some off-odor compounds and as an ecological parameter because it can be considered a determinative character of yeast evolution in natural fermentation. In fact, high acetoin producers (apiculate yeasts in the genera *Kloeckera* and *Hanseniaspora*) generally appear in the early stages of fermentation and possess a low fermentative power. In contrast, *Saccharomyces cerevisiae* and other wine yeasts with high fermentation power generally produce low acetoin levels (61) and are able to synthesize large amounts of higher alcohols and ethanol, metabolizing the large quantities of acetoin produced by apiculate yeasts (86). It could be hypothesized that acetoin, a flavorless compound that affects the oxidation-reduction balance of yeast metabolism, controls the extent to which flavor compounds, such as diacetyl, are present in wine. As pyruvate is converted into a variety of end products, including ethanol and acetoin, the low-ethanol-tolerant yeasts, such as apiculate yeasts (41, 47), could preferentially switch pyruvate utilization to the acetoin biosynthetic pathway in order to dispose of toxic amounts of ethanol. This hypothesis is consistent with the results reported by Tsau et al. (79), which indicated that the conversion of pyruvate to acetoin is a mechanism of detoxification in *Lactobacillus plantarum.*

The biochemical and genetic studies of the phenotype "high acetoin production" in *Saccharomyces* species could lead to a most important alternative technology. Thus, considering acetoin to be a probable metabolism regulator of some products of must fermentation, the character "acetoin production" could be introduced in the strain selection program for industrial applications.

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